nature portfolio

Peer Review File

Targeting chromosomally unstable tumors with a selective KIF18A inhibitor

Editorial Note: This manuscript has been previously reviewed at another journal that is not operating a transparent peer review scheme. This document only contains reviewer comments and rebuttal letters for versions considered at *Nature Communications*.

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The authors did not address the comments related to the demonstration of differential efficacy of the KIF18A inhibition in vivo. I disagree with the contention that "it is difficult to justify from a time, cost and ethics standpoint to use animals to confirm a negative in vitro result". In my mind, important controls are very easy to justify, and are even necessary to convincingly make a major claim of the study.

In addition, and perhaps more importantly, my additional request was to also demonstrate the results in a "real" mouse model (that is, beyond a xenograft model), which I thought was necessary for Nature Cancer, but is perhaps beyond the scope for Nature Communications. Since one of the major selling points of this manuscript is the in vivo demonstration of KIF18A inhibition, the lack of mouse models or PDXs, as well as lack of data demonstrating CIN-dependent sensitivity in vivo, diminish some of the enthusiasm.

On the positive side, the revised manuscript did improve the molecular characterization of KIF18A inhibition. The most interesting data that were added are: (a) an analysis of the interactions between KIF18A inhibition and SAC activity (dual inhibition experiment); and (b) following the molecular response of a non-sensitive cell line (CAL51) to the KIF18A inhibitor. These additions improved the manuscript in my mind.

A few remaining comments:

(1) The authors did not address my comment related to the link between mitotic duration and KIF18A response. This is a shame given recent suggestions that the response to the drug is largely determined by the length of mitosis (and that CIN/aneuploidy/WGD are merely proxy of that).

(2) The authors did not address my comment related to the in vitro testing of their KIF18A inhibitor on non-transformed hematopoietic/neural human cell lines. These are relatively easy experiments to do, and the mouse data (mentioned in the authors' response) do not address this point in a satisfactory manner.

(3) The authors interpret their results with the combined MPS1 and KIF18 inhibition as

evidence that cell lines that are insensitive to KIF18A may have SAC deficiencies. I think this assertion needs to be toned down bit – these data certainly support the notion that SAC activity is important for the response to KIF18A inhibition, but does not necessarily suggest a deficient SAC in insensitive cell lines.

Reviewer #2:
Remarks to the Author:
The authors report VLS-1272 as a new inhibitor for KIF18A. In vitro studies show that VLS-1272 can inhibit KIF18A's activity. VLS-1272 is claimed to block KIF18A localisation at the kinetochore. Other inhibitors for KIF18A exist, but VLS-1272 is a new addition to this toolset. In cells, the inhibitor induces mitotic arrest but whether it affects KIF18A specifically is unclear. KIF18A's mechanism of action in cells is known and so they need to show evidence for VLS1272 disrupting KIF18A's known in vivo function. The Xenograft experiments are an important aspect of drug development - but without an inv ivo mechanism of action, it is not clear if the effects reported are specific to KIF18A inhibition. In general, there are a variety of VLS-1272 treatment experiments being reported, but each of them has its unique caveats and slightly contradicting observations, making it an overall weak manuscript.

Other major concerns:
Figure 1: The authors use information from Cancer Dependency Map to confirm that Kif18A is a vulnerability of chromosomally unstable cancer cells. However, this information is already known through other means and so there is not much novelty in this opening figure. Lack of novelty is fine if the authors could include some information on spindle checkpoint or DNA checkpoint status in these datasets (a negative result would also be useful for readers). The table related to the figure needs improvement. At the moment, it looks like a weak justification for the entire work.

Figure 2: 'Micronuclei' can be tolerated well in cell lines. Nuclear atypia of other kinds - binucleation or multinucleation or lagging strands of DNA (as in these examples, PMID: 15843429 PMID: 17346968) could be more useful to assess (they are easy to assess using DAPI). In summary, the authors focus on micronuclei to interpret CIN status is somewhat misleading without consideration of other types of segregation defects. CIN has to be directly inferred to conclude on CIN decrease or increase (WGS is one possibility).

Figure 3: The data presented here show novel findings on VLS-1272, and relates it to the existing inhibitor BTB-1. However, in the rest of the manuscript, there is no comparison of VLS-1272 with any of the other existing inhibitors of KIF18A. This is a large gap

because it will be difficult for the field to position VLS-1272 against any of the already existing tools to abrogate the Kif18A function. How different is KIF18A-IN-1 (sold by MedChemExpress as KIF18A inhibitor) different from VLS1272 - are they identical with different activities or accessibility etc should be considered since the chemical structures (Figure 3A and MCE website) are similar but not identical.

Figure 4: This figure compares the consequence of KIF18B and MPS1 inhibitor treatment. Without a clear mechanism of action for KIF18B inhibitor, its hard to comprehend what these differences mean. Uncelar how they can compare Figure 4A outcome in different cell types with those in the rest of the figure. Figure 4H and 4I do not seem to agree with each other (2nM Bay-1217389) values are unclear.

Figure 5 would be valuable if the authors can compare against other KIF18A inhibitors. Volasertib, Irinotecan, Doxorubicin are all drugs with unrelated mechanism of action. Even if VLS-1272 does not outperform other KIF18A inhibitors in the market, it will be important information for the field to know.

Figure 6: Experiments here do not show that VLS-1272's action in cells is specifically through KIF18A. KIF18A's mechanism of action at kinetochore-microtubule attachment is known well, but the authors do not explore whether the inhibitor blocks these events. Kif18a influences kinetochore-microtubule attachments Clasp1-Astrin-Kif2b pathway (PMID: 20852589). Astrin-SKAP is a known marker for bioriented and end-on attached kinetochores (PMID: 31808746) and I am surprised that the authors are not checking Astrin localisation following inhibition to establish that their IF studies on KIF18a localisation following inhibition are trustworthy. Without an explanation of the underpinning process, the work is not mature enough for Nature Communications.
Figure 7: The Xenograft work is important and in panel E, the authors report an increase in mitotic arrest which provides some strength tho their hypothesis, however these information in the absence of cellular specificity (Figure6) can not be interpreted strongly. Also, in the Tumor Xenograft images (panel C) the authors observe an increase of KIF18A on spindles (is this different from what was obtained in cells; Fig S3A)?

Discussion:
1.KIF18a Ko mice show micronuclei and infertility - aren't the authors concerned about clinical trials?
2. Checkpoint proteins Bub1, BubR1 and several other kinetochore proteins are all phospho-regulated and their phosphorylation status (using antibodies) can readily provide insight into attachment status. Even if not experiments per se, they should at least discuss the possibility of checking these phosphorylation levels to fully understand the impact of the inhibitor in CIN-positive and negative cells. This is important because it can help explain why the inhibitor affects CIN cells specifically.

Other minor points.
1. Repeats are missing (biological/technical repeats must be differentiated and appropriate repeats must be considered. Few examples figure 2G, 2I, and 2C.
2. Immunoblots need uncropped blots and kDa (Fig 2D, 2F and 2K are all immunostained for KIF18A but the banding patterns are not comparable, making it challenging to link across experiments.
3. Discuss why Kif1A localisation (staining with DNA) is being measured (Fig 6B). Do they expect the protein to be on the chromatin? Need to use a kinetochore marker if the purpose is to measure kinetochore localisation. Figure 6A spindle pole and microtubule bound levels of KIF18A are also reduced.
4. Explain dots and crosses in Figure 3I. 3D, reeds repeats, 3E needs scale, explain if MTs are more stable in untreated controls.

Reviewer #3:
Remarks to the Author:
The manuscript describes the use of an inhibitor to further validate the kinesin-like protein KIF18A as an anticancer drug target. This is an important contribution to the literature supporting the development of KIF18A inhibitors. The authors have addressed in part the prior referees' comments.

The chemistry methods section added, which describes the synthesis of the study inhibitor is inadequate. The scheme (or Figure lacking a number) showing the synthesis of VLS-1272 shows labels a-h to indicate reagents and reaction conditions. These are not defined in any Figure legend: there is no figure legend. There is no descriptive discussion of the chemistry route to accompany the Figure. There is no acknowledgment as to prior work to prepare intermediates 3 and 7. These should include citation of prior patent applications (e.g. WO2018039386 for aldehyde 7) and journal articles (J. Med. Chem, 1999, 515 for carboxylic acid 3).

The chemistry methods section should indicate full details the method to determine purity (presumably LC of some sort). Chemical characterization of the key study inhibitor is superficial. MS data should be provided to 4 decimal places to provide some evidence of elemental composition. The 1H NMR data is predominantly reported as multiplet signals. Given the apparent complexity of the proton NMR spectrum, a 13C NMR should be provided as additional proof of structure. A figure of the actual spectrum would be helpful if anyone were to repeat this synthesis. As would a Figure of the HPLC chromatogram to provide clear evidence of purity.

Throughout the manuscript the details of the vehicle for formulation for use of VLS-1272 in PK, and antitumor animal experiments is not provided. This should be explicitly defined to allow some chance of this work being repeated.

Reviewer Responses to NCOMMS-23-08794A

Reviewer comments are in black text and author responses are in blue text

We thank the reviewers for their insightful comments and have detailed our actions and responses below. In this work, we have developed a potent, clinically translatable inhibitor of KIF18A and show that the mechanism and phenotypes observed with this inhibitor closely align with those observed with KIF18A loss-of-function studies published by multiple labs. This KIF18A inhibitor differentiates itself from other clinical anti-mitotic agents, relies on chromosome instability and the spindle assembly checkpoint for efficacy, and shows robust anti-tumor activity in xenograft models while sparing normal cell types, all of which have not been shown with other published KIF18A inhibitors. We hope the reviewers agree that the evidence provided supports these impactful conclusions.

Reviewer #1 (Remarks to the Author):

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In addition, and perhaps more importantly, my additional request was to also demonstrate the results in a "real" mouse model (that is, beyond a xenograft model), which I thought was necessary for Nature Cancer, but is perhaps beyond the scope for Nature Communications. Since one of the major selling points of this manuscript is the in vivo demonstration of KIF18A inhibition, the lack of mouse models or PDXs, as well as lack of data demonstrating CIN-dependent sensitivity in vivo, diminish some of the enthusiasm.

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We appreciate the reviewer's comments that the additional data included in the previous revision improved the manuscript.

A few remaining comments:

(1) The authors did not address my comment related to the link between mitotic duration and KIF18A

response. This is a shame given recent suggestions that the response to the drug is largely determined by the length of mitosis (and that CIN/aneuploidy/WGD are merely proxy of that).

As stated in our previous response (copied below), this is a interesting question that does warrant further study. We currently have an ongoing research collaboration aimed at addressing this specific hypothesis about mitotic duration. When completed, the results of the collaboration will be disclosed in a separate work. At this point, this further characterization is beyond the scope of this current study.

Previous response: We agree it would be interesting to explore mitotic duration, but measuring mitotic duration across a small panel of cell lines at the level needed for statistical significance would be a resource-intensive effort and is beyond the scope of this study.

(2) The authors did not address my comment related to the in vitro testing of their KIF18A inhibitor on non-transformed hematopoietic/neural human cell lines. These are relatively easy experiments to do, and the mouse data (mentioned in the authors' response) do not address this point in a satisfactory manner.

T cells are a non-transformed hematopoietic human cell type that was tested with VLS-1272 (Figure 4C), and no impact on proliferation was observed. As the reviewer mentioned, hematopoietic cell counts from mice treated with VLS-1272 were included in the revised manuscript. It is unclear why this data does not address the point in a satisfactory manner, as ex vivo culture of primary cells often involves artificial culture conditions, and data in an intact mouse is physiologically relevant. In addition, it is unclear why testing in these specific cell types (hematopoietic or neural cells) is preferable to testing in primary human T cells.

Reviewer's comment from prior round: (5) The authors tested several non-transformed cell lines, including T cells, for their response to the KIF18A inhibitor. Given the toxicity concern, and the fact that the prospect of reduced toxicity is a major potential advantage of KIF18A inhibitors, can the authors also test human hematopoietic and neural stem/progenitor cells?

In this reviewer's original comment (copied above), a question of toxicity was raised, which is a legitimate concern for many oncology therapies, and there are a huge range of interesting studies which could be proposed to investigate potential toxicities across a diversity of models However, as a molecule advances to the clinic, rigorous assessment of toxicity takes place during FDA-mandated GLP toxicology studies in pre-clinical studies, and then in humans during Phase 1 clinical trials. GLP 2-specieis animal toxicology studies (as well as other required studies) for Volastra's KIF18A inhibitor (which is derived from VLS-1272) have been completed and demonstrated sufficient preclinical safety to progress to an ongoing Phase 1 clinical study. While assessment in non-transformed hematopoietic or neural human cell lines using in vitro culture systems may provide some assessment of toxicity, we have cleared the high bar of the required studies for IND filing with the FDA.

(3) The authors interpret their results with the combined MPS1 and KIF18 inhibition as evidence that cell lines that are insensitive to KIF18A may have SAC deficiencies. I think this assertion needs to be toned

down bit – these data certainly support the notion that SAC activity is important for the response to KIF18A inhibition, but does not necessarily suggest a deficient SAC in insensitive cell lines.

We agree that the requirement of the SAC for KIF18A sensitivity does not necessarily imply that insensitive cell lines have SAC deficiencies. Any text suggesting that insensitive cells have SAC deficiencies has not been included in the manuscript. In our previous response to this reviewer's comment, we did suggest that insensitive cell lines may have SAC defects, but this statement does not appear in the manuscript text.

Reviewer #4 (Remarks to the Author):

The authors report VLS-1272 as a new inhibitor for KIF18A. In vitro studies show that VLS-1272 can inhibit KIF18A's activity. VLS-1272 is claimed to block KIF18A 4localization at the kinetochore. Other inhibitors for KIF18A exist, but VLS-1272 is a new addition to this toolset. In cells, the inhibitor induces mitotic arrest but whether it affects KIF18A specifically is unclear. KIF18A's mechanism of action in cells is known and so they need to show evidence for VLS1272 disrupting KIF18A's known in vivo function. The Xenograft experiments are an important aspect of drug development – but without an inv ivo mechanism of action, it is not clear if the effects reported are specific to KIF18A inhibition. In general, there are a variety of VLS-1272 treatment experiments being reported, but each of them has its unique caveats and slightly contradicting observations, making it an overall weak manuscript.

To address the reviewer's concern, we have included a new analysis (Figure 5A-B) which provides strong evidence that VLS-1272 acts through disrupting KIF18A. Sensitivity to VLS-1272 across >100 cell lines was compared with genome-wide gene essentiality in these cell lines. When VLS-1272 sensitivity was compared to the sensitivity profile of >16,000 genes, the top correlated gene by a large margin is KIF18A, for both CRISPR and RNAi-determine gene essentiality, indicating that the cell proliferation defects observed with VLS-1272 are through KIF18A.

It is unclear what the caveats and contradicting observations the reviewer is referring to. Known functions of KIF18A are listed below (with references). For each of these known functions (elucidated with genetic loss-of-function methods), we demonstrate that pharmacological KIF18A inhibition with VLS-1272 disrupt these known functions of KIF18A, both in vitro and in vivo:

- KIF18A is an ATPase (Mayr et al 2007, Catarinella et al, 2009). VLS-1272 inhibits the ATPase activity of purified KIF18A.
- KIF18A translocates on microtubules (Stumpff et al, 2011). VLS-1272 inhibits MT translocation activity of purified KIF18A.
- KIF18A is a dependency of chromosomally unstable cells (Marquis et al, 2021). VLS-1272 inhibits proliferation of CIN^{High} but not CIN^{Low} or normal cells. VLS-1272 also shows differential sensitivity in a CIN isogenic system, consistent with genetic data in the manuscript.
- KIF18A localizes to the mitotic spindle near the microtubule plus ends (Mayr et al, 2007, Stumpff et al 2008, others). VLS-1272 causes KIF18A to re-localize away from microtubule plus ends towards the spindle poles, in cell culture and in vivo.
- KIF18A controls chromosome congression (Mayr et al 2007, Stumpff et al 2008, others). VLS-1272 reduces chromosome congression, in cell culture and in vivo.
- KIF18A is involved in mitotic progression (Mayr et al 2007, others). VLS-1272 induces mitotic arrest, consistent with genetic data and other published KIF18A inhibitors (Tamayo et al, 2021), in cell culture and in vivo
- Loss of KIF18A results in micronuclei (Sepaniac et al, 2021). VLS-1272 induces micronuclei formation.
- KIF18A dependency relies on the spindle assembly checkpoint (Janssen et al 2018, Marquis et al 2021). VLS-1272 sensitivity is reduced with concurrent inhibition of the SAC.

A key piece of data demonstrating target engagement is the redistribution of KIF18A away from the microtubule plus-end, caused by disruption of the known MT translocation function of KIF18A (Figure 6A). The change in localization of KIF18A demonstrates that VLS-1272 is directly impacting the intended target. This relocalization is also observed in insensitive cells (Figure S3A-B) demonstrating that it is not an indirect effect of mitotic perturbation or arrest. Finally, relocalization of KIF18A has also recently been reported with a different KIF18A inhibitor, and the speed by which it happens indicates that the effect is direct (Schutt et al, Biorxiv, 2023).

Chromosome congression is another well-established function of KIF18A (Mayr et al, Curr Biol, 2007; Stumpff et al, Dev Cell, 2008; Liu et al, Genes & Cancer, 2010; others). VLS-1272 disrupts chromosome congression function both in vitro (Fig 6A, B, D) and in vivo (Figure 7 G-J). The loosening of the metaphase plate observed with VLS-1272 is consistent with KIF18A function and is not a phenotype and generally observed with anti-mitotics. The doses of VLS-1272 that inhibit the known functions of KIF18A (chromosome congression, mitotic progression) align with the doses that inhibit proliferation in those same cell lines.

Finally, we want to mention that a high related compound, VLS-1488, has progressed to Phase I clinical trials. The FDA accepted our claim that it is a highly specific KIF18A inhibitor with an almost identical data package.

Other major concerns:

Figure 1: The authors use information from Cancer Dependency Map to confirm that Kif18A is a vulnerability of chromosomally unstable cancer cells. However, this information is already known through other means and so there is not much novelty in this opening figure. Lack of novelty is fine if the authors could include some information on spindle checkpoint or DNA checkpoint status in these datasets (a negative result would also be useful for readers). The table related to the figure needs improvement. At the moment, it looks like a weak justification for the entire work.

The justification for this work is not only supported the analysis in Figure 1, but other high impact publications identifying KIF18A as a vulnerability of aneuploidy/whole genome doubled/chromosomally unstable cancer cells, as the reviewer referred to and as cited in the manuscript's introduction. Additional justification for the pursuit of KIF18A vs. other hits identified from the screen includes a high potential for druggability of KIF18A. We fully validate the therapeutic potential of KIF18A by creating a potent inhibitor suitable for cellular and in vivo use. These points were stated in the 2nd paragraph of the results section, copied below:

"Our internal findings were further corroborated by publications identifying KIF18A essentiality in models of aneuploidy, whole genome doubling, and CIN breast and colon cancer cells[18–20]. Based on these internal analyses, the independent identification of KIF18A by other groups, and the potential for therapeutic targeting of KIF18A by pharmacological inhibition, we focused on KIF18A in follow-up studies."

Figure 2: 'Micronuclei' can be tolerated well in cell lines. Nuclear atypia of other kinds -binucleation or

multinucleation or lagging strands of DNA (as in these examples, PMID: 15843429 PMID: 17346968) could be more useful to assess (they are easy to assess using DAPI). In summary, the authors focus on micronuclei to interpret CIN status is somewhat misleading without consideration of other types of segregation defects. CIN has to be directly inferred to conclude on CIN decrease or increase (WGS is one possibility).

To confirm that micronuclei levels correlate with CIN status specifically in the KIF2A-KIF2B cell line pair, we evaluated a more direct readout of CIN, defective anaphases (which includes lagging chromosomes and chromatin bridges). This new data in Figure 2B confirms that the KIF2A cell line exhibits higher levels of chromosome instability. This result is consistent with published data which we refer to in our previous response, copied below:

We agree that micronuclei levels alone do not necessarily indicate CIN. The KIF2A/KIF2B model of chromosome instability has been described in other publications which contain additional data demonstrating their altered CIN state as measured by an increase in chromosome missegregation and metastatic potential (Bakhoum et al, Nat Cell Biol, 2009; Bakhoum et al, Nature, 2018). We do agree that the outcomes in Figure 2C could be due to expression of the Kif2A/B proteins and not CIN, but other data in our paper (Figure 2) and other publications (Marquis et al, Nat Comm, 2021) support the connection with CIN and dependence on KIF18A beyond the KIF2A/2B model.

Figure 3: The data presented here show novel findings on VLS-1272, and relates it to the existing inhibitor BTB-1. However, in the rest of the manuscript, there is no comparison of VLS-1272 with any of the other existing inhibitors of KIF18A. This is a large gap because it will be difficult for the field to position VLS-1272 against any of the already existing tools to abrogate the Kif18A function. How different is KIF18A-IN-1 (sold by MedChemExpress as KIF18A inhibitor) different from VLS1272 - are they identical with different activities or accessibility etc should be considered since the chemical structures (Figure 3A and MCE website) are similar but not identical.

Other than BTB-1, the other existing inhibitors the reviewer is referring to (including KIF18A-IN-1) have been developed by Amgen and were published in Tamayo et al, J Med Chem, 2022. A compound derived from this work, AMG650, entered clinical trials in 2020. Since the submission of this manuscript, Volastra Therapeutics has licensed AMG650 from Amgen, and Volastra is now pursuing clinical development of AMG650 simultaneously with the clinical development of Volastra's internally-developed KIF18A inhibitor, of which VLS-1272 is derived.

Due to the parallel clinical trials, Volastra cannot compare the 2 clinical molecules or their precursors, which include VLS-1272 described in this manuscript, and the compounds described in Tamayo et al, 2022. Premature comparison of these non-clinical compounds could potentially mislead patients and clinical investigators as to which molecule is "better" and create bias in clinical trial enrollment. Our top priority is getting these drugs to patients, and the clinical trials will determine which drug provides the most benefit to patients. We hope the reviewer agrees that the ultimate goal of this research is to impact the lives of patients, and we are in the rare and exciting situation where the clinical needs must take precedent to these preclinical questions.

Figure 4: This figure compares the consequence of KIF18B and MPS1 inhibitor treatment. Without a clear mechanism of action for KIF18B inhibitor, its hard to comprehend what these differences mean.

A KIF18B inhibitor is not evaluated in this work.

The mechanism of the KIF18A inhibitor is detailed in Figures 3 and 6 and discussed in detail in the first paragraph of responses to this reviewer. These data show that the KIF18A inhibitor VLS-1272 inhibits the ATPase activity of KIF18A and prevents the translocation of KIF18A along microtubules. This immobilization results in a mislocalization of KIF18A during mitosis, leading to defective chromosome congression and mitotic arrest. The mechanism of how SAC loss (which we achieved through MPS1 inhibition) affects dependency on KIF18A has also be described in other publications (Janssen et al 2018; Marquis et al 2021).

Unclear how they can compare Figure 4A outcome in different cell types with those in the rest of the figure.

Figure 4A is a data summary showing the anti-proliferative effect of VLS-1272 in 7 different cell lines. Figure 4A includes cell lines used in all other parts of the figure such as JIMT-1 (4D), CAL51 (4E and 4G) and OVCAR-3 (4F and 4G), so they can be directly compared. For example, the EC50 proliferation for OVCAR-3 in Figure 4A is 10.9 nM. Correspondingly, Figure 4F shows that cell death is not observed below the EC50 (4.5 nM), but it observed above the EC50 (40 nM), demonstrating that the anti-proliferative effect in OVCAR-3 observed in Figure 4A is likely due to cell death caused by VLS-1272. Cell line names have been added to the graphs to make the comparison clearer.

Figure 4H and 4I do not seem to agree with each other (2nM Bay-1217389) values are unclear.

Figures 4H (HCC15) and 4I (HCC1806) are data from 2 different cell lines, both shown to be sensitive to VLS-1272 in Figure 4A. As they are different cell lines, the data are not expected to be identical, but they both show the same trend that weakening the SAC through MPS1 inhibition decreases sensitivity to VLS-1272.

Figure 5 would be valuable if the authors can compare against other KIF18A inhibitors. Volasertib, Irinotecan, Doxorubicin are all drugs with unrelated mechanism of action. Even if VLS-1272 does not outperform other KIF18A inhibitors in the market, it will be important information for the field to know.

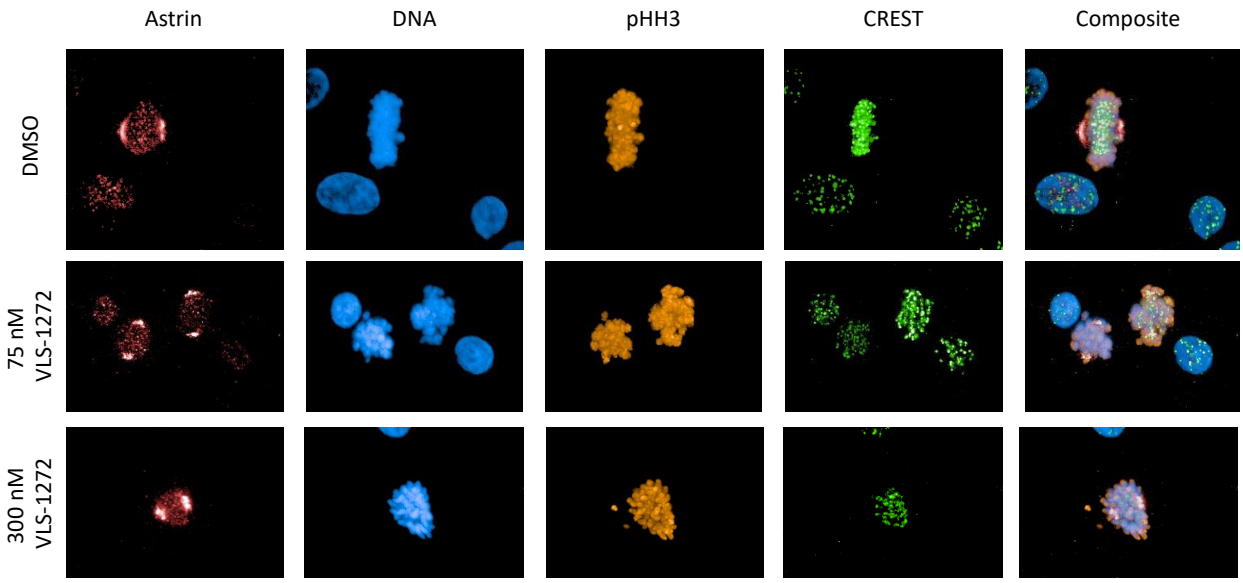
Please see our response to Figure 3 regarding comparison to other KIF18A inhibitors in the market. The field will soon know the performance of two KIF18A inhibitors currently in the clinic, which is the critical data needed for patients.

Figure 6: Experiments here do not show that VLS-1272's action in cells is specifically through KIF18A. KIF18A's mechanism of action at kinetochore-microtubule attachment is known well, but the authors do not explore whether the inhibitor blocks these events. Kif18a influences kinetochore-microtubule attachments Clasp1-Astrin-Kif2b pathway (PMID: 20852589). Astrin-SKAP is a known marker for bioriented and end-on attached kinetochores (PMID: 31808746) and I am surprised that the authors are not checking Astrin localisation following inhibition to establish that their IF studies on KIF18a

localisation following inhibition are trustworthy. Without an explanation of the underpinning process, the work is not mature enough for Nature Communications.

The reviewer has suggested that staining for Astrin at the kinetochores in the presence or absence of VLS-1272 would further confirm the specificity of KIF18A inhibition. However, upon reviewing the literature, there is evidence that the effects of KIF18A loss on Astrin localization as shown in Manning et al (PMID: 20852589) are likely context-dependent, as Janssen et al (PMID: 30122526) evaluated Astrin localization in the context of KIF18A loss and observed that Astrin remained at kinetochores, in contrast to results from Manning et al.

To address the reviewer's concern, we stained for Astrin in the presence of KIF18A inhibition (and CREST as kinetochore marker) and observed that Astrin localization was maintained at kinetochores in the presence of VLS-1272 (below). Our results are consistent with that observed by Janssen et al. The discrepancy observed between Manning et al and Janssen et al indicate that there are complexities in the relationship between KIF18A and Astrin that are likely dependent on choice of cell line and potentially other factors. It is beyond the scope of this work to further understand the details their regulation.



Regarding whether VLS-1272's action is specifically through KIF18A, please see our response to the first paragraph of Reviewer #4's comment. VLS-1272 changes the localization of mitotic KIF18A, resulting in phenotypes (defective chromosome congression, mitotic arrest, micronuclei formation, sensitivity of CIN^{High} cells, dependence on an intact SAC) that are unique to and identical to those observed with KIF18A knockdown. Additionally, new analysis included in Figure 5A-B further confirms on-target effects of VLS-1272, as the pharmacologic sensitivity to a large panel of cell lines to VLS-1272 is extremely correlated to the cells genetic dependency to KIF18A, an extremely high bar for specificity.

Finally, it is unclear why this reviewer considers our KIF18A localization data not trustworthy. We present visual evidence of the mis-localization in Figures 6A and S3A, quantify the mis-localization with VLS-1272 in Figure 6B and S3B, and demonstrate that the dose at which KIF18A mis-localization is observed (above

13.7 nM) correlates very well with the anti-proliferation IC50 observed in a sensitive cell line (16.3 nM). The localization of KIF18A shown is consistent with published reports, and the change in localization with VLS-1272 also aligns with published data demonstrating the dependence of KIF18A localization on it's motor activity (Stumpff et al, Dev Cell, 2009). Additionally, a preprint published during the revision process (Schutt et al, Biorxiv, 2023) shows that mitotic KIF18A localization before and after treatment with the Amgen KIF18A inhibitor very closely mimics the localization we observe with VLS-1272. The relocalization of KIF18A in Schutt et al happens within a matter of minutes, demonstrating that inhibitor effects are direct.

Figure 7: The Xenograft work is important and in panel E, the authors report an increase in mitotic arrest which provides some strength tho their hypothesis, however these information in the absence of cellular specificity (Figure6) can not be interpreted strongly. Also, in the Tumor Xenograft images (panel C) the authors observe an increase of KIF18A on spindles (is this different from what was obtained in cells; Fig S3A)?

The increase in mitotic arrest (as measured by phospho-Histone H3 in Figure 7K) does support known mechanism of KIF18A inhibition, aligns with what is observed in cultured cancer cells (Figure 6E), and has also been reported for other KIF18A inhibitors (Figure 6B in Tamayo et al, J Med Chem, 2022). This consistency between studies, despite different inhibitors being used, supports that this result is an on-target readout of KIF18A inhibition. It is unclear why the increase in mitotic arrest in the xenograft tumors cannot be "interpreted strongly", and it is unclear what the "absence of cellular specificity" is referring to. If they are referring to on-target effects of KIF18A, please see our response to the first paragraph of Reviewer #4.

In the original Figure 7C, it does appear there is an increase of KIF18A on mitotic spindles and we thank the reviewer for pointing out this image. After reviewing the data, we realized this image was from an earlier experiment where staining intensity does not appear to be matched in the panels, and after reviewing multiple images, was not consistent with the data seen more generally. We have replaced Figure 7C with images that are more representative of the results from this study, and as expected, the data are more consistent with those in 7E. The in vivo data of KIF18A localization in Figure 7C-F is consistent with what is shown in cells (Figure 6 A-B), that treatment with VLS-1272 shifts KIF18A localization away from the MT plus ends and towards the spindle poles.

Discussion:

1.KIF18a Ko mice show micronuclei and infertility - aren't the authors concerned about clinical trials?

A potential concern about micronuclei induced by KIF18A loss of function is related to genome instability that may lead to cancer. It has previously been shown that KIF18A loss-of-function mice have increased levels of micronuclei (Fonseca et al, JCB, 2019). However, these mice do not have increased incidence of cancer, due to the finding that micronuclei induced by KIF18A loss in normal cells are more stable compared to micronuclei generated by other means (Sepaniac et al, JCB, 2021). Furthermore, equal chromosome segregation was maintained in KIF18A-deficient cells with no evidence of aneuploidy (Fonseca et al, JCB, 2019). These data demonstrate that genome instability is not a consequence of

KIF18A loss, despite the incidence of micronuclei. Thus, the standard evaluation of genotoxic potential using FDA-compliant tests should be sufficient to warrant progression of a KIF18A inhibitor. Notably drugs that induce micronuclei are acceptable for oncology indications, and PARP-inhibitors, which have positively impacted the lives of countless cancer patients, are known to induce micronuclei (see for example the NDA reports for Olaparib at https://www.accessdata.fda.gov/drugsatfda_docs/nda/2014/206162Orig1s000PharmR.pdf)

Infertility was observed in KIF18A loss-of-function mice due to germ cell defects (Czechanski et al, Dev Biol, 2015). Risk of infertility is acceptable for oncology clinical trials. There are many approved cancer therapies linked to risk of infertility, and potential for infertility is not considered a major obstacle in oncology drug development given the life-threatening potential of most cancers. The statement below from an FDA publication demonstrates fertility is not a major concern for therapies being developed for advanced cancers.

"A study of fertility and early embryonic development is not warranted for pharmaceuticals intended for the treatment of patients with advanced cancer"

From FDA publication: Oncology Pharmaceuticals: Reproductive Toxicity Testing and Labeling Recommendations Guidance for Industry (https://www.fda.gov/media/124829/download)

2. Checkpoint proteins Bub1, BubR1 and several other kinetochore proteins are all phospho-regulated and their phosphorylation status (using antibodies) can readily provide insight into attachment status. Even if not experiments per se, they should at least discuss the possibility of checking these phosphorylation levels to fully understand the impact of the inhibitor in CIN-positive and negative cells. This is important because it can help explain why the inhibitor affects CIN cells specifically.

Loss of KIF18A and inhibition by BTB-1 does increase phosphorylation of kinetochore proteins such as Hec1 (Kemura et al, JCB, 2021). The reviewer raises a good suggestion that examining phosphorylation status of kinetochore proteins can inform on the kinetochore- microtubule attachment status and that it would be interesting to look at the impact of VLS-1272 in this context. We have included in the discussion (5th paragraph) how CIN-positive vs. CIN-negative cells may respond differently to VLS-1272 and included this point about examining attachment status through evaluation of kinetochore protein phosphorylation to understand differences in responses between.

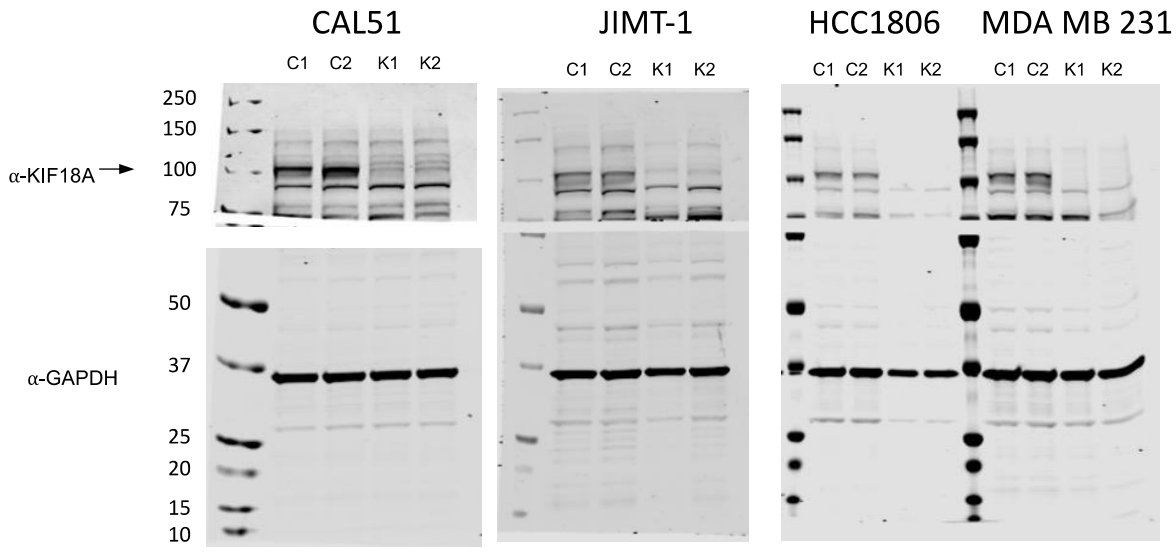
Other minor points.
1. Repeats are missing (biological/technical repeats must be differentiated and appropriate repeats must be considered. Few examples figure 2G, 2I, and 2C.

Thank you for pointing out this omission. Replicates and error bars, and description of the repeat types have been included for these and other experiments.

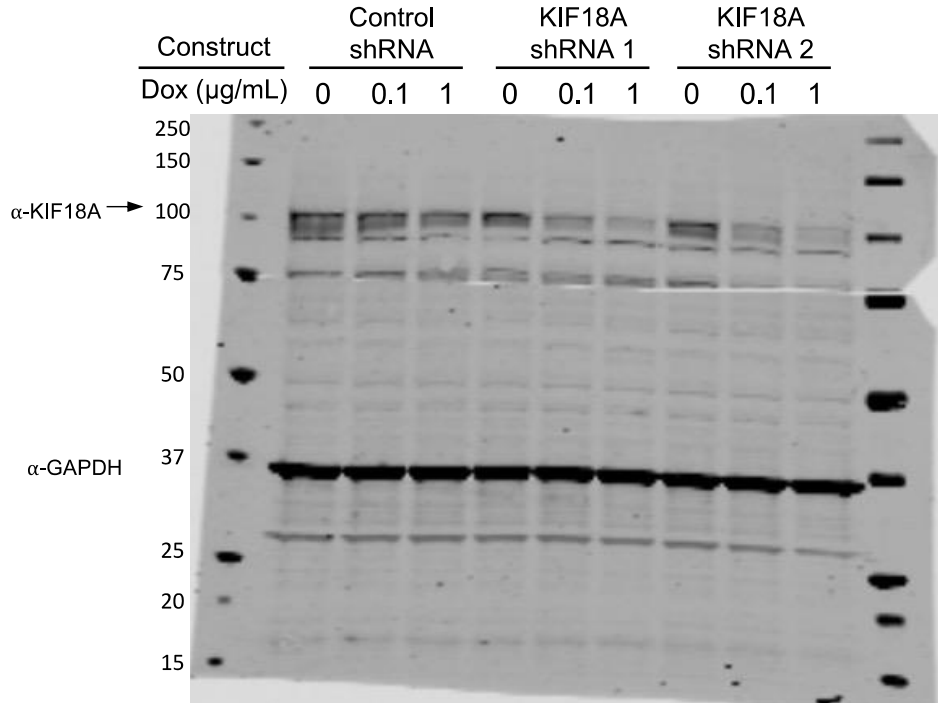
2. Immunoblots need uncropped blots and kDa (Fig 2D, 2F and 2K are all immunostained for KIF18A but the banding patterns are not comparable, making it challenging to link across experiments.

Banding patterns are dependent on factors such as the type of gel used and cell line. Uncropped blots were included in the previous response to reviewer's comments and are copied again below. We confirm that these images adhere to Nature Portfolio's image integrity policy (as required for all Nature portfolio publications), and as according to that policy, will be provided upon request.

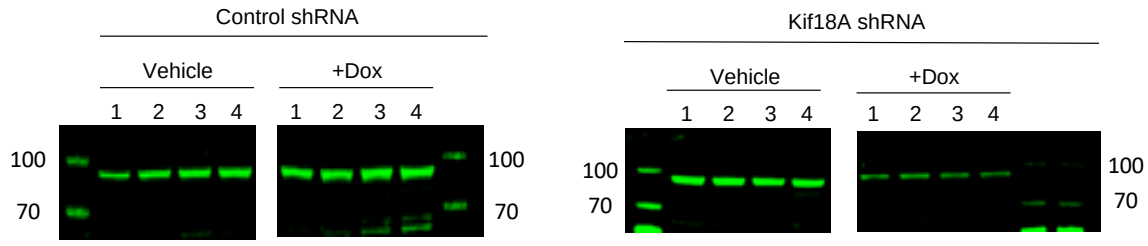
Blots from Figure 2E:



Blots from Figure 2G:

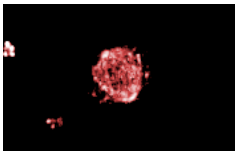


Blots from Figure 2L:

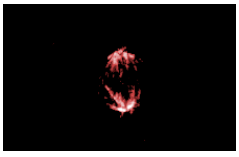


3. Discuss why Kif1A localisation (staining with DNA) is being measured (Fig 6B). Do they expect the protein to be on the chromatin? Need to use a kinetochore marker if the purpose is to measure kinetochore localisation. Figure 6A spindle pole and microtubule bound levels of KIF18A are also reduced.

Kif1A localization was not measured. Kif18A localization was measured to illustrate that localization changes in the presence of inhibitor treatment, and that data provide strong evidence that the in vitro mechanism observed of VLS-1272 inhibiting the motility of KIF18A is also observed in cells. Kif18A is a kinesin protein that travels towards the plus end of microtubules. In untreated cells, KIF18A is localized along the microtubule spindle, not only at kinetochores, as displayed in Figure 6A and below.



In VLS-1272 treated cells, the presence of the inhibitor prevents KIF18A from translocating along microtubules towards the plus end. In a mitotic spindle, the plus ends are located near the DNA, whereas the minus ends are located near the spindle pole. VLS-1272 causes relocalization close to the minus ends of the microtubule. This change in localization can be quantified by measuring the % of signal co-localized with DNA (near the plus end of the microtubule), normalized to the % co-localizing with alpha-tubulin. Figure 6B quantifies this ratio and demonstrates that VLS-1272 treatment results in KIF18A staining away from the plus end, due it it's inability to translocate towards the plus end (shown in Figure 3E).



4. Explain dots and crosses in Figure 3I.

The dots and crosses are explained in the Figure Legend, copied below:

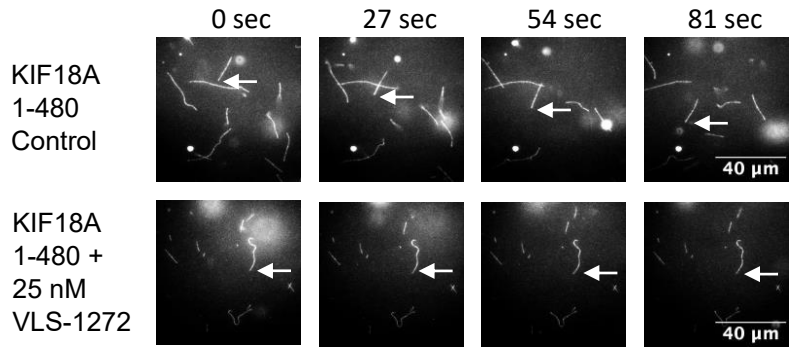
Each dot represents one compound. Compounds with poor permeability are denoted by "x".

3D, reeds repeats,

3D has been replaced by a figure which contains repeats and error bars

3E needs scale, explain if MTs are more stable in untreated controls.

Figure 3E contains a 40 um scale bar, copied below. Each panel in the figure is the same scale.



In Figure 3E, microtubule motility was measured, not microtubule stability. The arrows point to microtubules moving along a “surface” of immobilized KIF18A via KIF18A’s motor domain, which can also be visualized in the supplemental videos provided. In this assay, microtubules are stabilized with taxol to prevent MT depolymerization and allow visualization of MT filaments.

Reviewer #5 Replacement for Reviewers #2 & #4 (Remarks to the Author):

The manuscript describes the use of an inhibitor to further validate the kinesin-like protein KIF18A as an anticancer drug target. This is an important contribution to the literature supporting the development of KIF18A inhibitors. The authors have addressed in part the prior referees' comments.

The chemistry methods section added, which describes the synthesis of the study inhibitor is inadequate. The scheme (or Figure lacking a number) showing the synthesis of VLS-1272 shows labels a-h to indicate reagents and reaction conditions. These are not defined in any Figure legend: there is no figure legend. There is no descriptive discussion of the chemistry route to accompany the Figure. There is no acknowledgment as to prior work to prepare intermediates 3 and 7. These should include citation of prior patent applications (e.g. WO2018039386 for aldehyde 7) and journal articles (J. Med. Chem, 1999, 515 for carboxylic acid 3).

The chemistry methods section should indicate full details the method to determine purity (presumably LC of some sort). Chemical characterization of the key study inhibitor is superficial. MS data should be provided to 4 decimal places to provide some evidence of elemental composition. The 1H NMR data is predominantly reported as multiplet signals. Given the apparent complexity of the proton NMR spectrum, a 13C NMR should be provided as additional proof of structure. A figure of the actual spectrum would be helpful if anyone were to repeat this synthesis. As would a Figure of the HPLC chromatogram to provide clear evidence of purity.

Throughout the manuscript the details of the vehicle for formulation for use of VLS-1272 in PK, and antitumor animal experiments is not provided. This should be explicitly defined to allow some chance of this work being repeated.

Although we had used characterization for tool compounds in this journal as guides for the level of detail to provide for this report, the reviewer's suggestions are valid, and we have addressed them.

Due to conformers, the ^{1}H NMR of this class of compounds does not allow us to describe the coupling patterns even more precisely even with high-temperature NMR. We have added the ^{13}C NMR summary, along with images of the ^{1}H (rt and 80 C) and ^{13}C NMRs in Figures S8-10. We have provided HRMS data and the HPLC trace for VLS-1272 (Figure S7). Since we had solved a single-crystal X-ray structure that confirms the structure of VLS-1272, we have also included these data in the Supplementary Material (Figure S11).

The synthetic Scheme in the Supplementary Material was updated with a Figure notation.

The formulation procedure has also been provided in the Methods.

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
Reviewer #1:

The authors did not add any experiments or analyses to address any of my previous comments, so I have nothing new to add. It is at the Editors' discretion whether to accept the manuscript to Nat Commun without the full resolution of these issues.

Reviewer #1's assessment of the response to Reviewer #4's comments:

I generally agree with the comments previously made by Reviewer #4.

The authors satisfactorily addressed the first point related to the specificity of the new inhibitor, I think that their response to this point is compelling.

Figure 1: I agree with the authors that the rational for focusing on KIF18A is clear, although I agree with Reviewer #4 that this is mostly coming from others' work and the relevant analysis presented in the current manuscript is not novel.

Figure 2: The authors addressed this point properly. I think that the link to CIN is sufficiently strong.

Figure 3 and Figure 5: The justification for the lack of comparison to the other compounds currently undergoing clinical trials is completely understandable. However, the fact remains that the manuscript doesn't include any comparisons to other inhibitors of KIF18A, which could have been of much interest for the field. The authors' statement "the field will soon know the performance of two KIF18A inhibitors currently in the clinic, which is the critical data needed for patients" is not really helpful.

Figure 4: The authors address this point in a satisfactory manner.

Figure 6: The authors address this point in a satisfactory manner, I am not concerned about the KIF18A localization data.

Figure 7: I believe that the authors did not understand the Reviewer's comment, and that it's actually very similar to mine – the in vivo experiments do not compare sensitive to insensitive cells and do not demonstrate CIN-dependent sensitivity in

vivo. I think that this is what Reviewer $4 meant by "absence of cellular specificity" – the molecular determinants of KIF18A sensitivity, studied in vitro, are not really studied in vivo. The second point has been addressed properly.

Discussion points: The authors have addressed these points properly.

Minor points: All of the minor points have been addressed properly.

Reviewer #5:
Remarks to the Author:
The comments from my previous review have been addressed fully.

Dear Editor and Reviewers,

Thank you for the opportunity to re-submit our manuscript to Nature Communications. This revised manuscript addresses the Reviewer's concerns regarding benchmaking to other KIF18A inhibitors by including comparisons to the recently published KIF18A inhibitor AM-1882. The new data and comparisons included in the manuscript demonstrate that VLS-1272 and AM-1882 have similar properties including potency and selectivity profiles, and both display noncompetitiveness with ATP and slow binding kinetics. Both compounds show similar binding poses when docked into the KIF18A homology model. Furthermore, both compounds show similar effects in cells such as changes in KIF18A spindle localization and increases in mitotic index.

We have incorporated the Reviewer's follow-up suggestions by including a KIF18A inhibitor comparison table and additional discussion. The new data and comparison of other key properties of VLS-1272 and AM-1882 are summarized in Table S3. Furthermore, we have included in the discussion the limitation of not having in vivo validation of the CIN mechanism of action without in vivo comparison to a CIN^{Low} group.

Given the excitement around KIF18A and the progression of KIF18A inhibitors through clinical trials, we see these benchmarking comparisons as providing timely and relevant context important to understanding the effects of pharmacological inhibition of KIF18A. We believe that inclusion of these items will further illustrate how VLS-1272 is an important advancement to the KIF18A field. We hope that with these revisions, the manuscript will be deemed suitable for publication at Nature Communications.

Kind regards,
Christina Eng (on behalf of the authors)

Reviewer Comments (in black) **and Responses** (in blue)

Reviewer #1 (Remarks to the Author):

Reviewer #1:

The authors did not add any experiments or analyses to address any of my previous comments, so I have nothing new to add. It is at the Editors' discretion whether to accept the manuscript to Nat Commun without the full resolution of these issues.

Reviewer #1's assessment of the response to Reviewer #4's comments:

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Figure 2: The authors addressed this point properly. I think that the link to CIN is sufficiently strong.

Figure 3 and Figure 5: The justification for the lack of comparison to the other compounds currently undergoing clinical trials is completely understandable. However, the fact remains that the manuscript doesn't include any comparisons to other inhibitors of KIF18A, which could have been of much interest for the field. The authors' statement "the field will soon know the performance of two KIF18A inhibitors currently in the clinic, which is the critical data needed for patients" is not really helpful.

We appreciate the understanding of our justification regarding the lack of comparison for clinical compounds, and we also understand the importance of demonstrating how VLS-1272 compares to other inhibitors of interest to the field.

To address this point, we have included the following data comparing the preclinical KIF18A inhibitors VLS-1272 and AM-1882.

- A new study directly comparing potency, ATP-competitiveness, and time-dependence of VLS-1272 vs. AM-1882 inhibition of the KIF18A ATPase domain. This data is shown in Figure 3B vs. Figure S1B, Figure S1C and summarized in Table S3. VLS-1272 and AM-1882 show similar potencies, both are non-competitive with ATP, and both exhibit slow-binding kinetics with an improvement in potency over time.

- Docking of VLS-1272 and AM-1882 into a KIF18A homology model that demonstrates both molecules bind to KIF18A at the same interface. This data is shown in Figure S2.
- Comparison of cell line sensitivity to VLS-1272 vs. AM-1882 (using data published for AM-1882). This analysis demonstrates that there is high overlap of cell line sensitivity between the compounds (Figure S1E and Table S5)
- Table S3 and discussion in the manuscript text highlighting the following similarities between VLS-1272 (as disclosed in the manuscript) and AM-1882 (as published):
    - Selectivity against other kinesins
    - Lack of sensitivity of normal proliferating T cells to KIF18Ai
    - Changes in cellular KIF18A localization at the mitotic spindle
    - Increase in mitotic index

Figure 4: The authors address this point in a satisfactory manner.

Figure 6: The authors address this point in a satisfactory manner, I am not concerned about the KIF18A localization data.

Figure 7: I believe that the authors did not understand the Reviewer's comment, and that it's actually very similar to mine – the in vivo experiments do not compare sensitive to insensitive cells and do not demonstrate CIN-dependent sensitivity in vivo. I think that this is what Reviewer $4 meant by "absence of cellular specificity" – the molecular determinants of KIF18A sensitivity, studied in vitro, are not really studied in vivo. The second point has been addressed properly.

We appreciate Reviewer #1 clarifying this comment by Reviewer #4. To highlight this point identified by both reviewers, we added text discussing the limitation of not having an in vivo validation of the CIN dependency of KIF18A inhibitors (Discussion section, end of 2nd paragraph). We also added a reference (Payton et al, Nature Cancer, 2023) that published differential effects to KIF18A inhibitors in vivo based on CIN status.

Discussion points: The authors have addressed these points properly.

Minor points: All of the minor points have been addressed properly.

Reviewer #5 (Remarks to the Author):

The comments from my previous review have been addressed fully.

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The authors have addressed the major concerns. I have no further comments.